Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Micronucleus investigation of alcoholic patients with oral carcinomas

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Genet. Mol. Res. 1 (3): 246-260 (2002)
Received May 5, 2002
Published September 23, 2002

ABSTRACT. The micronucleus test (MN) is used as an indicator of genotoxic exposition, since it is associated with chromosome aberrations. An increased mutation rate in oral squamous cells, indicated by an increased MN frequency, is also related to the development of oral carcinomas. We evaluated the frequencies of MN and other metanucleated anomalies in the buccal squamous cells of 30 alcoholics with oral or oropharyngeal carcinomas, and compared them to a control group of abstinent health individuals. Microscopic examination was made of 2000 cells per individual from each of three distinct areas of the mouth: around the lesion (A), opposite to the lesion (B) and in the upper gingival-labial gutter (C); C was used as a control region because of low tumor frequency. There was a seven-fold increase in MN frequency in region B, a three-fold increase in region A and a two-fold, though nonsignificant, increase in C; indicating a gradient of frequencies towards carcinogenesis: C $\rightarrow$ A $\rightarrow$ B. Comparisons of frequencies of various types of metanucleated cells: binucleated, karyorrhexis (KR), karyolysis (KL) and broken egg (BE) in patients and controls showed, with few exceptions, highly significant differences. This gave us a better understanding of the dynamics of this squamous epithelium, supporting a more efficient biomonitor based on these various metanucleated anomalies: the repair index $RI = (\overline{KL} + \overline{KR}) / (\overline{MN} + \overline{BE})$. Also, the apparently contradictory results from regression analysis revealed that the MN frequency decreased with age and alcohol consumption, probably

because of slow cell proliferation, and consequently led to a loss of homeostasis due to aging. In addition, in the analysis of nonparametric variables only one CAGE question was significant, confirming the effect of alcohol. In conclusion, the MN test and the repair index could be used for monitoring clinical evolution, by means of intra- and inter-individual cellular comparisons, in subjects with healed or surgically removed tumors or leukoplastic lesions, after chemo- or radiotherapeutic treatments.

Key words: Micronuclei, Alcohol, Oral cancer, Nuclear anomalies, Cancer genetics

INTRODUCTION

A micronucleus (MN) is a small extranucleus separated from the main one, generated during cellular division by late chromosomes or by chromosome fragments. Because of its association with chromosomal aberrations, MN have been used since 1937 as an indicator of genotoxic exposition based on the radiation studies conducted by Brenneke and Mather (Heddle et al., 1983). Since then, many other studies have been made with plants and animals, as well as with humans, both *in vitro* and *in vivo* (Evans, 1997). Investigations on MN frequencies support the widely accepted assumption that MN are a product of early events in human carcinogenic processes, especially in oral regions (Stich et al., 1982a, 1988; Stich and Rosin, 1984; Dave et al., 1992; Benner et al., 1994a; Ghose and Parida, 1995; Desai et al., 1996; Roberts, 1997), especially because they are virtually absent in unexposed mucosa (Lippman et al., 1990).

It was found many years ago that alcohol has mutagenic, carcinogenic and teratogenic effects (Obe and Ristow, 1979). More recent studies have revealed mutagenic effects of alcohol on human chromosomes *in vitro* (Gattás et al., 1989) and *in vivo*, even after a long period of abstinence (Gattás and Saldanha, 1997). Moreover, Stich and Rosin (1983) also observed an increased MN frequency related to the mutagenic effects of alcohol in buccal mucosal cells of alcoholic smokers.

Alcohol consumption and smoking appear to be the two most frequent exogenic risk factors for the development of oral cancer (Seitz et al., 1998). Alcoholic beverages, acting locally, allow other carcinogenic substances to pass into target cells and also systemically lead to decreased cell metabolism, producing a relative immune deficiency (cf. Carvalho, 1997). However, specific dietary components, such as β-carotene, retinol, α-tocopherol, vitamin A, riboflavin, zinc, selenium, turmeric oil and Chinese tea, have consistently been proved to confer protection from, and even remission of the carcinogenic process, based on MN frequencies (Stich et al., 1984; Benner et al., 1994b; Prasad et al., 1995; Hastak et al., 1997; Li et al., 1999).

Epidemiological studies have often reported abusive and/or chronic alcohol consumption as well as smoking habits to be associated with increased occurrence of different kinds of oral carcinoma (Franco et al., 1989; Boffetta et al., 1992; Maier et al., 1992; Kabat et al., 1994; MacFarlane et al., 1995). Also, an increased MN frequency was found among smokers and/or tobacco chewers with oral carcinomas (Stich et al., 1982b; Adhvaryu et al., 1991; Tolbert et al., 1991).

Consequently, the MN test, currently known as the micronucleus assay (Obe and Natarajan, 1990), has been used for screening populations under risk of mutagenic agents that cause oral neoplasias (Stich and Rosin, 1984; Garewal et al., 1993; Desai et al., 1996; Kumar et al., 2000), especially for the identification of pre-clinical steps of the carcinogenic process (Ramirez et al., 1999).

We report a study of alcoholic patients with oral or oropharyngeal carcinomas, evaluating frequencies of MN and other metanucleated anomalies in their oral mucosal squamous cells, compared to a control group of abstinent, healthy individuals.

MATERIAL AND METHODS

Subjects

There were 30 patients, aged from 37 to 76 years old, with oral or oropharyngeal carcinomas, whose alcohol and tobacco consumption had begun from four to 61 years before, and a control group of 30 individuals, abstinent due to their religious convictions. Patients and controls were matched for social-economic status. Three distinct regions in the mouth of each individual were examined. In the patients, the analysis was made on the side opposite to the lesion (A) around the lesion (B), and in the upper gingival-labial gutter (C), often used as a control region because of the low occurrence of tumors. Correspondingly, controls had cells collected from the right (D) and left (E) cheeks, and also from the upper gingival-labial gutter region (F).

Cytogenetic analysis

The study of buccal squamous cells was performed by the standard technique of Sarto et al. (1987), with some modifications made for the requirements of this investigation as follows: the oral mucosas were scraped gently with a cytobrush and the material was submersed in 5 ml of saline solution (0.9% NaCl). After centrifugation (10 min at 1500 rpm), the pellet was fixed in 3:1 methanol/acetic-acid once, or twice if necessary, for 5 min. Cool and wet glass slides were used for the final cell suspension, after the material was stained with Fuelgen nuclear reaction plus 1% fast green stain. Permanent slides were prepared for a microscopic "blind-test" analysis, made according to Tolbert et al. (1992). The frequency of MN was evaluated as total number of MN (TMN) and number of cells with MN (CMN) per individual because some cells have more than one MN. The MN next to the main nucleus, as well as those in binucleated cells, were also taken into account. Furthermore, metanucleated anomalies other than MN, such as binucleated cells (BI), karyorrhexis (KR), karyolysis (KL) and broken eggs (BE) were also taken into account (Figure 1).

Intervening factors

It was possible to identify independent variables theoretically expected to be associated with the occurrence of MN (dependent variables), based on anamnestic interviews of the patients, parental consanguinity, familial recurrence, smoking habit, alcohol consumption, psychiatric hospitalization and neoplasia site; these were classified as qualitative (nonparametrical)

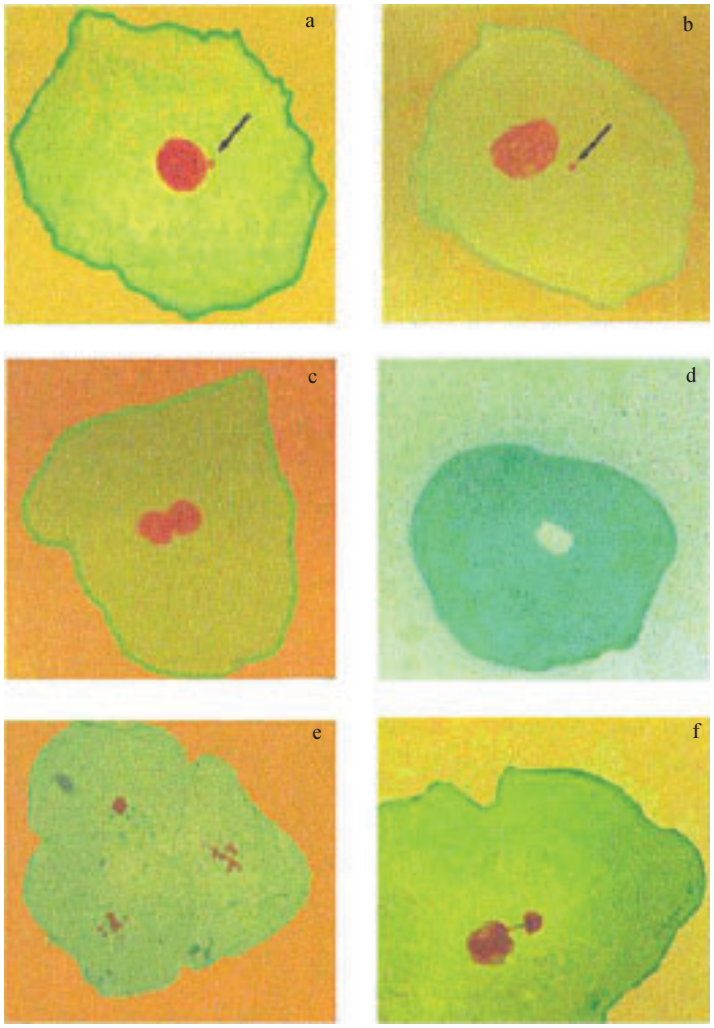

Figure 1. Metanucleated anomalies: micronuclei (a,b); binucleated cell (c); karyolysis (d); karyorrhexis (e) and broken egg (f).

variables, indirectly related to the metanucleated cells. However, age of patients, age at the beginning of alcohol consumption, age by the end of alcohol consumption, time (duration) of alcohol consumption and time of smoking habit were considered as quantitative (parametric) variables, directly associated with the metanucleated events.

The alcohol dependence of patients was diagnosed by the CAGE (Cut-down, Annoyed, Guilt, Eye-opener) questionnaire (Ewing, 1984). The application of the four questions of CAGE was performed on the basis of previous experience with alcoholics (Gattás and Saldanha, 1993).

Statistical analysis

Two thousand cells were analyzed per individual in each of the three distinct regions of the mouth of 30 patients and 30 controls, so that 60,000 cells were investigated in each oral area, totaling 180,000 in each group or 360,000 cells altogether. The sample number of 2000 cells should give an untruncated spontaneous dispersion of MN events. The distribution of all

types of metanucleated cells departed very significantly (P<0.001) from the expected normal dispersion (χ^2 values from 18 to 150), with high variance among patients. In the controls, the MN frequency fit a Poisson distribution quite well (P ≅ 1.00) and the occurrence of other metanucleated cells, except for BE, apparently followed a binomial distribution, all positively asymmetrical. Consequently, intra- and interindividual comparisons were made by means of nonparametric tests, such as Mann-Whitney, Kruskal-Wallis and Dunn's multiple comparison (nonparametric ANOVA) tests. The Student *t*-test was used for age comparisons. The role of nonparametric intervening factors was analyzed by means of 2 x 2 recurrence tables through Fisher's exact method, in which subjects were classified to have MN or not. The MN distributions of patients grouped by each of two-class factors were compared with the Mann-Whitney test.

The quantitative (parametric) factors were analyzed by means of multiple regression analysis of the dependent variables (MN and other metanucleated cells), tentatively normalized by square root transformation ($\sqrt{y + 0.5x1000}$), of those intervening factors.

The critical level for rejection of the null hypothesis was considered to be 5%. Detailed information about sampling as well as cytological and statistical procedures are reported elsewhere (Ramirez, 2000).

RESULTS

Intra- and interindividual comparisons

The distribution of age, duration of smoking habit and alcohol consumption in years as well as the frequencies of TMN (CMN) per 2000 cells per individual in the regions A, B and C among 30 patients as well as in regions D, E and F among 30 controls were recorded (Table 1). The difference (14.5 years) between average age of patients (52.9 ± 1.6) and controls (38.4 ± 1.5) was highly significant (P<0.0001). Cells with MN counts higher than one were predominant in the patients, apparently as a result of the clastogenic action of alcohol. These patients also had TMN frequencies as high as seven. On the other hand, controls with two or more MN per cell were found only twice. Moreover, younger patients had more TMN than older ones, contrary to the expected "age effect".

The number of TMN and CMN per 2000 cells per individual was determined for each oral region of the patients and controls (Figure 2). The dispersion (variance) of TMN was higher than that of CMN. Consequently, TMN appears to be more efficient than CMN for monitoring carcinogenic processes. A comparison was made of TMN frequencies in the three oral regions of the patients through the Kruskal-Wallis test (Table 2). First, the three regions were compared directly and, then comparisons were made indirectly, after estimating individual differences between each of the three pairwise comparisons in order to improve the power of the test. Estimates were obtained by means of direct and indirect (based on interindividual differences) analysis.

The frequency of TMN in the three oral regions of patients revealed a highly significant heterogeneity (P = 0.005). The pairwise comparison, B *vs* C, was significant (P<0.01) but the comparisons between A *vs* B and A *vs* C were not (P>0.05). Comparisons of pairwise interregional oral differences (indirect analysis) of TMN frequencies (A-B, A-C, B-C), increased the significance levels of the results for regional heterogeneity (P = 0.0003) and increased the significance of the comparison of B *vs* C (P<0.001); the comparison A *vs* B also became significant

Table 1. Distribution of age, smoking habit and alcohol consumption as well as TMN frequency per 2000 cells per individual in A (near an oral carcinoma), B (in the cheek opposite an oral carcinoma) and C (control labial-gingival gutter) oral regions of 30 patients and in D, E and F (corresponding to A, B and C) oral regions of 30 controls, with their respective means and standard errors.

Individual	Patients						Controls			
	Age	sh	ac	TMN			Age	TMN		
				A	B	C		D	E	F
1	67	na	49	3	7(4)	1	33	1	0	0
2	53	na	43	2	5(4)	0	40	0	0	1
3	47	29	25	1	2(1)	0	32	1	0	0
4	50	na	16	2	2	0	41	0	0	0
5	49	41	38	0	1	0	38	0	0	0
6	52	38	33	1	1	0	52	1	0	0
7	52	15	04	0	0	0	34	0	0	0
8	48	23	20	2	4(2)	5	50	0	0	0
9	47	32	29	4	1	1	33	0	0	0
10	67	57	56	2	3	0	51	2	2	0
11	55	40	33	0	0	0	42	0	0	1
12	37	30	18	0	5	1	30	0	0	0
13	57	47	47	0	2	0	33	1	0	1
14	40	26	15	0	6(4)	3	32	0	0	1
15	48	33	21	0	2(1)	0	36	0	1	0
16	69	61	55	1	0	0	42	0	0	0
17	37	28	27	3	0	1	35	0	1	0
18	55	36	36	2	1	1	29	0	1	0
19	50	33	23	1	4(3)	0	31	0	1	0
20	54	20	23	0	0	0	28	0	1	1
21	52	34	32	0	0	0	50	0	0	0
22	50	35	25	0	1	0	40	1	1	0
23	54	47	24	0	0	0	33	0	0	0
24	54	40	38	0	0	4(2)	64	0	0	0
25	42	37	30	0	2	0	32	0	0	0
26	54	39	28	2	1	1	30	0	0	2
27	76	58	59	1	0	0	47	0	0	0
28	61	42	41	0	0	0	35	0	0	0
29	61	42	42	0	3	0	36	0	0	0
30	49	34	29	1	6(5)	0	42	1	0	0
mean ± SEM	52.90 ± 1.63	36.93 ± 2.09	31.97 ± 2.38	0.93 ± 0.21	1.97 (1.57) ± 0.39 (0.29)	0.60 (0.53) ± 0.23 (0.20)	38.37 ± 1.55	0.27 ± 0.09	0.27 ± 0.09	0.23 ± 0.09

	TMN distribution (see Figure 1)							
		n				n		
	N	A	B	C	N	D	E	F
sh, smoking habit (years)	0	15	10	21	0	23	23	24
ac, alcohol consumption (years)	1	6	6(8)	6	1	6	6	5
-, not available	2	6	5(4)	0(1)	2	1	1	1
MN, micronuclei	3	2	2(3)	1	-	-	-	-
( ), cells with MN	4	1	2(3)	1(0)	-	-	-	-
n, number of individuals	5	0	2	1	-	-	-	-
N, MN frequency	6	0	2(0)	0	-	-	-	-
T, total number of MN	7	0	1(0)	0	-	-	-	-
	T	28	59(47)	18(16)	T	8	8	7

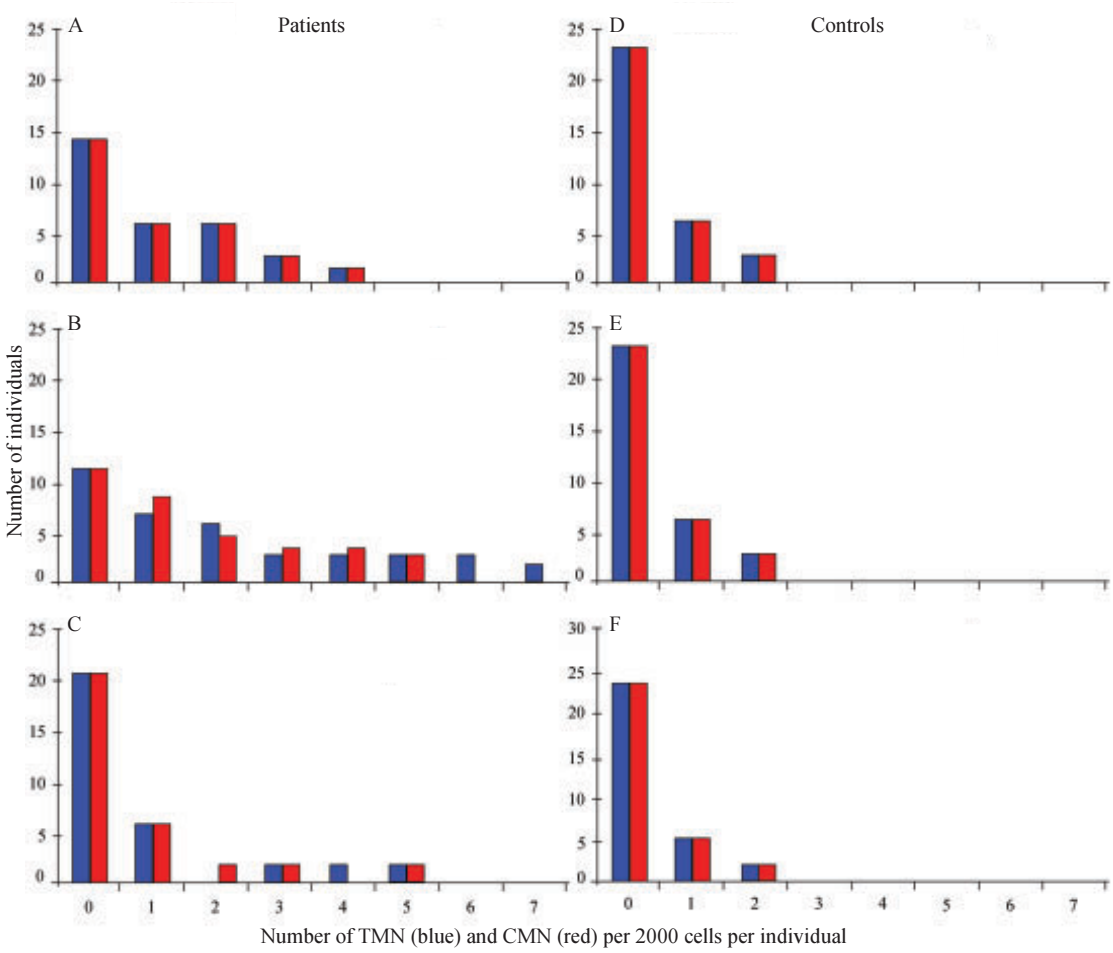

Figure 2. Distribution of total number of micronuclei (TMN) and number of cells with micronuclei (CMN) per 2000 cells per individual among oral regions A, B and C of 30 patients as well as in D, E and F oral regions of 30 controls. For A-F abbreviations, see legend to Table 1.

($P<0.05$). However, a strong degree of homogeneity ($P = 0.943$) was found with nonparametric analysis of variance of the TMN distribution in the three oral regions of the controls. All oral regions were quite homogeneous in the controls and consequently interregional pairwise comparisons by Dunn's multiple comparison test could not be applied to the data (Table 2).

A comparison was made of MN frequencies between patients and controls, as well as their relative distributions; comparisons were also made of other metanucleated events such as BI, KL, KR and BE with the Mann-Whitney test (Table 3). MN differences were highly significant ($P<0.001$) for tumoral region and significant for the region opposite to the lesion ($P = 0.030$) but not for the upper gingival-labial gutter ($P = 0.445$), in comparison with the controls. There was also a seven-fold increase in the frequency of MN in the region around the lesion (1.97 ± 0.39), a three-fold increase in the opposite region (0.93 ± 0.21) and a two-fold but nonsignificant increase in the upper gingival-labial gutter (0.60 ± 0.23), when compared to the

Table 2. Direct and indirect* nonparametric analysis of variance (Kruskal-Wallis) of the total number of micronuclei (TMN) per 2000 cells per individual among oral regions A, B and C of 30 patients as well as among oral regions D, E and F of 30 controls through Dunn's multiple comparison test.

Patients			
Region	TMN (direct) mean ± SEM	Region	TMN (indirect) mean ± SEM
A	0.933 ± 0.209 (0.933 ± 0.209)	A-B	-1.033 ± 0.400 (-0.633 ± 0.334)
B	1.966 ± 0.388 (1.566 ± 0.294)	A-C	0.333 ± 0.281 (0.400 ± 0.252)
C	0.600 ± 0.228 (0.533 ± 0.202)	B-C	1.366 ± 0.402 (1.033 ± 0.327)
Significance	H (χ^2) = 10.47; P = 0.0053 (H (χ^2) = 9.53; P = 0.0085)		H (χ^2) = 16.50; P = 0.0003 (H (χ^2) = 13.03; P = 0.0015)
Dunn´s multiple comparison test			
Comparison (direct)	P	Comparison (indirect)	P
A x B	>0.05 (>0.05)	(A-B) x (A-C)	<0.05 (<0.05)
B x C	<0.01 (<0.01)	(A-B) x (B-C)	<0.001 (<0.01)
A x C	>0.05 (>0.05)	(A-C) x (B-C)	>0.05 (>0.05)
Controls			
Region	TMN** direct		
D	0.266 ± 0.095		
E	0.266 ± 0.095		
F	0.233 ± 0.092		
Significance	H (χ^2) = 0.12; P = 0.9428		

Estimates for number of cells with MN per individual (CMN) in parentheses. * Based on pairwise differences between oral regions of patients. ** Identical values of TMN and CMN were found in controls. For abbreviations A-F, see legend to Table 1.

mean frequency in the controls (0.25 ± 0.31), revealing a gradient of frequencies (C → A → B). Moreover, there were highly significant differences between patients and controls in the frequencies of metanucleated cells in the three oral regions, except for BE frequencies in all oral regions and for KR frequency in the upper gingival-labial gutter (Table 3).

Intervening factors

The effect of age on the frequencies of metanucleated cells was investigated among both patients and controls through regression analysis; however, none of the regression coefficients were significant. Contrary to what was expected, MN frequencies were not significantly associated with the age of subjects; most of the correlation coefficients were negative. Square root transformation of the dependent variables produced significant correlations for KL (r = -0.387) and KR (r = -0.358).

A stepwise multiple regression analysis was made by considering parametric anamnestical data as independent variables (Table 4). Significant results were found in the regressions of the frequencies of metanucleated anomalies per 2000 cells per individual for the following variables: age of patients, age at the beginning of alcohol consumption, age at the end of alcohol consumption and time of alcohol consumption. Tentative normalization of dependent

Table 3. Comparison of number of cells with micronuclei (CMN), total number of micronuclei (TMN) and metanucleated anomaly (BI, KR, KL, BE) frequencies per 2000 cells per individual, among A, B and C oral regions of 30 patients and D, E and F oral regions of 30 controls, through the Mann-Whitney test.

Variables	Patients (mean ± SEM)			Controls (mean ± SEM)			Difference between means			U (Mann-Whitney) (P)		
	A	B	C	D	E	F	A-D	B-E	C-F	A-D	B-E	C-F
CMN	0.93 ± 0.21	1.57 ± 0.29	0.53 ± 0.20	0.27 ± 0.09	0.27 ± 0.09	0.23 ± 0.09	0.67	1.30	0.30	304.5 (0.0297)	219.0 (0.0006)	399.5 (0.4454)
TMN	0.93 ± 0.21	1.97 ± 0.39	0.60 ± 0.23	0.27 ± 0.09	0.27 ± 0.09	0.23 ± 0.09	0.67	1.70	0.37	304.5 (0.0297)	211.5 (0.0004)	399.0 (0.4409)
BI	11.47 ± 1.60	9.93 ± 1.51	11.03 ± 2.98	3.10 ± 0.33	2.53 ± 0.44	3.23 ± 0.48	8.37	7.40	7.80	177.5 (<0.0001)	183.0 (<0.0001)	262.0 (0.0055)
KR	2.23 ± 0.98	1.77 ± 0.63	2.57 ± 1.28	5.27 ± 0.93	6.03 ± 1.04	1.83 ± 0.60	-3.03	-4.27	0.73	190.5 (0.0001)	186.0 (<0.0001)	363.5 (0.1985)
KL	18.63 ± 3.89	17.90 ± 3.40	20.57 ± 3.93	4.20 ± 1.33	1.37 ± 0.34	4.20 ± 1.49	14.43	16.54	16.4	226.0 (0.0009)	135.5 (<0.0001)	146.5 (<0.0001)
BE	0.27 ± 0.12	0.20 ± 0.07	2.50 ± 1.68	0.43 ± 0.24	0.33 ± 0.21	1.33 ± 0.93	-0.17	-0.13	1.17	447.0 (0.9694)	426.0 (0.7170)	430.0 (0.7671)
Repair index $RI = (\overline{KL} + \overline{KR})/(\overline{MN} + \overline{BE})$	17.4	9.1	7.5	13.5	12.3	3.8	-	-	-	-	-	-

MN, micronuclei; BI, binucleated cells; KR, karyorrhexis; KL, karyolysis; BE, broken eggs. For A-F abbreviations, see legend to Table 1.

variables by means of square root transformation produced similar results for TMN and CMN, so that only the former was taken into account.

All regression coefficients that associated the age of the patients and alcohol consumption with MN frequency were small and negative, but were significant. Besides MN, only KL frequency was significantly inversely correlated with the beginning of alcohol consumption and positively with the end of alcohol consumption at the same level of probability, principally when the data were transformed through square root transformation. However, a positive association ($r = 0.38$) was found only for the carcinogenic area, when estimated with the same transformation (Table 4).

Dichotic comparisons of nonparametric independent variables with MN frequencies, through contingency and Mann-Whitney tests, were not significant, except for CAGE diagnosis for alcoholism. When we examined the four questions of CAGE, only one of them (*Feel need to cut down on drinking?*), estimated through the Mann-Whitney test ($U = 4.0$; $P = 0.052$), was significant, confirming the primary effect of alcohol on MN occurrence.

Table 4. Stepwise multiple regression analysis of frequencies of total number of micronuclei (TMN), number of cells with micronuceli (CMN) and other metanucleated anomalies (karyolysis, KL) per 2000 cells per individual, upon statistically significant independent variables of 30 patients.

Dependant variable (y)	Oral region	Transformation (y)	Mean ± SD	Independent variable (x)	$b \pm s_b$	t	P	r^2
TMN (CMN)*	A	NTD	0.778 ± 1.086	ea	-0.018 ± 0.009	4.297	0.043	0.1467
TMN (CMN)	B	NTD	1.667 ± 1.901	ap	-0.081 ± 0.039	4.371	0.047	0.1488
TMN (CMN)	C	NTD	0.630 ± 1.305	ea	-0.029 ± 0.010	8.037	0.009	0.2509
				ta	-0.041 ± 0.018	4.809	0.038	0.1669
					a = 2.804 ± 1.144; F = 4.934; P = 0.016			
TMN (CMN)	C	SRT	958.85 ± 466.53	ap	-21.898 ± 8.858	6.112	0.021	0.2030
				ea	-9.478 ± 3.452	7.539	0.011	0.2390
					a = 2399.12 ± 399.26; F = 5.749; P = 0.009			
KL	A	NTD	19.814 ± 22.101	ba	-1.324 ± 0.539	6.029	0.021	0.1943
KL	B	SRT	3562.00 ± 2164.76	ea	35.707 ± 17.330	4.245	0.050	0.1452
KL	C	SRT	4141.22 ± 2306.82	ba	-125.400 ± 57.458	4.763	0.039	0.1600

*TMN becomes significant or slightly increases the significance of the estimated regression coefficients for CMN; NTD, untransformed data; SRT, square root transformation ($\sqrt{y + 0.5}x1000$); ea, end of alcohol consumption; ap, age of patient; ta, time *(duration) of alcohol consumption; ba, beginning of alcohol consumption. For A-C abbreviations, see legend to Table 1.

DISCUSSION

Intraindividual comparisons between the three oral regions of patients revealed extremely significant differences, with a three-fold increase in the tumorous region (B) and a one and a half fold increase on the side opposite to the lesion (A), compared to the upper gingival-labial gutter (C). In controls, however, the MN frequencies in both cheeks (D and E) were quite similar and not significantly different in comparison with the F region.

The gradient of MN frequencies in the oral regions of patients (C → A → B) was confirmed in the comparisons with the controls, revealing a seven-fold increase in the MN frequency in the region around the lesion, a three-fold increase in the opposite region and a two-fold but nonsignificant increase in the upper gingival-labial gutter (Table 3). Stich and Rosin (1983) also reported a six-fold increase in the MN frequency of alcoholic smokers when compared to individuals that were strictly alcoholics or smokers, revealing a synergistic effect.

During the malignant process, the path to carcinogenesis appears to be correlated with increasing MN frequencies. This idea is supported by the similar MN frequencies found in the controls, as well as by the investigations of Stich et al. (1982a,b), Stich and Rosin (1983), Tolbert et al. (1991), Benner et al. (1994a) and Kumar et al. (2000).

The gradient of MN frequencies among patients could be explained by the assumption that C is a region relatively unexposed to mutagenic agents, while the A region, exposed similarly to B but not tumorized, represents an intermediate carcinogenic step; apparently B has the lowest biological threshold for mutagenic agents, such as alcohol and tobacco.

Moreover, studies on metanucleated anomalies other than MN are scarce, and most of

them are related to cell injury, cell death and mitotic errors. Also, some authors have failed to find significant associations of these metanuclear anomalies with apoptosis and carcinogenesis mechanisms (Nakano and Oka, 1991; Tolbert et al., 1991, 1992).

Increased frequencies of KL anomalies, also significant in the multiple regression analysis (Table 4), occur in the pre-keratinization process, which represents an adaptive response to cellular injuries (Pindborg et al., 1980). This anomaly is also evident in necrotic cells (Wyllie, 1981) and is related to cytotoxicity (Tolbert et al., 1991). In addition, it must be assumed that this anomaly is intrinsic to the squamous epithelium, especially because of the chronic effect of the masticatory process on the oral mucosa. Probably, the constant action of mutagenic agents such as alcohol and tobacco increases the rate of cellular deaths, as indicated by the significant increase of this anomaly in the patients (Table 3).

Since KR and BE should be considered as previous morphological stages of KL and MN, respectively, in squamous epithelium, these metanucleated formations were analyzed together for nuclear degeneration following the stages: clastogenesis → mutagenesis → carcinogenesis, during the BE → MN → KR → KL degenerative process, supporting homeostasis of the oral epithelial tissue. Consequently, a repair index (Table 3), represented by $RI = (\overline{KL} + \overline{KR}) / (\overline{MN} + \overline{BE})$, should reflect the dynamics of carcinogenesis. It revealed that KL increases and MN decreases with age in the cancer patients (Table 4). In fact, production of KL and KR anomalies should represent the repair process due to intercellular selection, occurring before cytogenetic damages, as indicated by BE and MN structures (Figure 3). A different way to evaluate the carcinogenesis process in oral cancer based on metanucleated anomalies was recently proposed by Bhattathiri (2001).

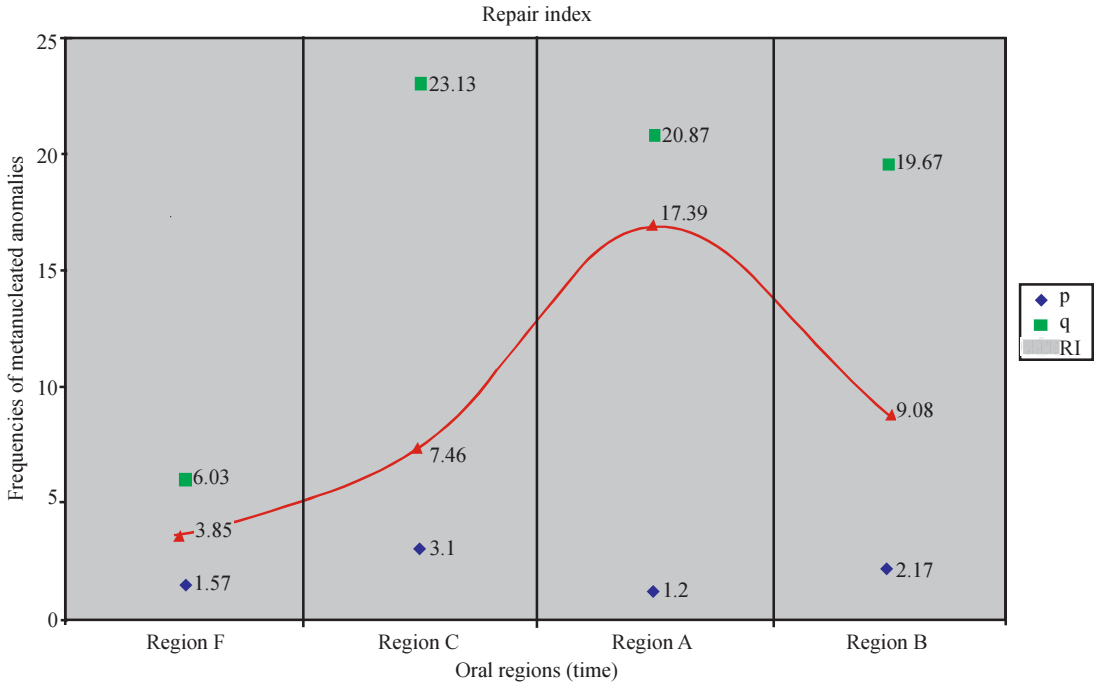

Figure 3. Interaction between frequencies of metanucleated anomalies as measured by micronuclei (MN) plus broken eggs (BE) (p) corresponding to nuclear damage and karyolysis (KL) plus karyorrhexis (KR) (q) revealing cellular repair through the index: $RI = (\overline{KL} + \overline{KR}) / (\overline{MN} + \overline{BE})$. For A-C and F abbreviations, see legend to Table 1.

Assuming that the mean of the F oral region of controls indicates a spontaneous MN frequency in the absence of clastogens, and that the oral regions (C → A → B) of patients represent different progressive stages in the dynamics of the carcinogenic process (Figure 3), it is likely that, after exposure to clastogens, the occurrence of KL plus KR increases abruptly after exposure, but steadily decreases thereafter. The MN plus BE frequencies are unstable around 2.0, because there is an initial tendency to produce abortive MN (BE) as the process reaches a critical stage, from which point carcinogenesis becomes disruptive, corresponding herein to RI ≅ 9.0 in the B region (Figure 3).

Different from what was expected, none of the frequencies of MN and other metanucleated cells were significantly correlated with age among patients and controls. It has often been reported that MN frequency (Kayal et al., 1993; Ghose and Parida, 1995), structural chromosome aberrations (Gattás and Saldanha, 1997) and neoplasias (Tan et al., 2001; Llewellyn et al., 2001) increase with age. However, most of the correlation coefficients were unexpectedly negative and nonsignificant, except for KL ($r = -0.387$) and KR ($r = -0.358$) patterns in the upper gingival-labial gutter of controls revealing that both frequencies of these anomalies diminish, probably because of a decrease in cellular proliferation and repair efficiency due to aging (Lofti and Machado-Santelli, 1996).

Although regression analysis gave consistent results, Table 4 revealed some contradictory situations. It is expected that age of patients and age by the end of alcohol consumption (indirectly) and time (duration) of alcohol consumption (directly) will increase and age at the beginning of alcohol consumption will decrease with the amount of alcohol consumption associated with the metanuclear formations. However, the small but significant regression coefficients for MN occurrence (Table 4) mean that the frequencies diminished irregularly after the critical stage of disruptive carcinogenesis, the situation in which most of patients were found when examined. An opposite explanation would support the negative regression coefficients for KL frequencies found in the oral region B of the patients, where there were also small but significant positive regression coefficients, as was expected.

Results from regression analysis of the cancer patients could be explained by the assumption that the MN frequency, due to exposure to alcohol, increased strongly, early, but decreased afterwards, and remained at a level significantly greater than before alcohol consumption, causing apoptosis (Slomiany et al., 1998; Castaneda and Kinne, 2001; Wu and Cederbaum, 2001) before carcinogenesis, while KL frequency conversely increased significantly from MN and other metanucleated transformations during the repair process. The sequential phases of RI described in Figure 3 should be a rough extrapolation, since they only took into account three steps in the oral regions of the patients. Finally, analysis of non-parametric synergistic variables revealed that only one question of the CAGE questionnaire, related to the intention to stop drinking, was significant enough to detect differences in MN frequencies between patient groups, indirectly revealing the effect of alcohol.

In conclusion, although it is not pathognomonic, the MN test is a simple, practical, inexpensive and non-invasive screening technique for clinical prevention and management of subjects under carcinogenic risks, after exposure to genotoxic agents or situations, such as abusive and chronic consumption of alcohol, tobacco and/or other mutagenic drugs, or professional manipulation of derivatives of petroleum and other toxic industrial substances. The MN test, and consequently the repair index, might be useful for monitoring the clinical evolution of subjects with healed or surgically removed tumors or leukoplastic lesions, after chemo- or

radiotherapeutic treatments, by means of intra- and interindividual cellular comparisons.

ACKNOWLEDGMENTS

The authors are very grateful to Dr. Marcos Brasilino de Carvalho of the Serviço de Cirurgia da Cabeça e Pescoço of the Hospital Heliópolis for clinical evaluation of patients and valuable medical assistance. They are also indebted to Dr. Gilka Jorge Figaro Gattás for allowing us to perform this research at the Laboratório de Citogenética of the Departmento de Medicina Legal, Ética Médica, Medicina Social e do Trabalho of the Universidade de São Paulo under grant LIM-42 of this Medical School. Research supported by Conselho Nacional de Pesquisas Científicas e Tecnológicas (CNPq) (grant No. 133947/95-5).

REFERENCES

Adhvaryu, S.G., Dave, B.J. and **Trivedi, A.H.** (1991). Cytogenetic surveillance of tobacco-areca nut (mava) chewers, including patients with oral cancers and premalignant conditions. *Mutat. Res. 261*: 41-49.

Benner, S.E., Lippman, S.M., Wargovich, M.J., Lee, J.J., Velasco, M., Martin, J.W., Toth, B.B. and **Hong, W.K.** (1994a). Micronuclei, a biomarker for chemoprevention trials: results of a randomized study in oral pre-malignancy. *Int. J. Cancer 59*: 457-459.

Benner, S.E., Wargovich, M.J., Lippman, S.M., Fisher, R., Velasco, M., Winn, R.J. and **Hong, W.K.** (1994b). Reduction in oral mucosa micronuclei frequency following alpha-tocopherol treatment of oral leukoplakia. *Cancer Epidemiol. Biomarkers Prev. 3*: 73-76.

Bhattathiri, V.N. (2001). Amitotic cell divisions and tumour growth: an alternative model for cell kinetic compartments in solid tumours. *Oral. Oncol. 37*: 288-295.

Boffetta, P., Mashberg, A., Winkelmann, R. and **Garfinkel, L.** (1992). Carcinogenic effect of tobacco smoking and alcohol drinking on anatomic sites of the oral cavity and oropharynx. *Int. J. Cancer 52*: 530-533.

Carvalho, M.B. (1997). História natural do câncer da cavidade oral. In: *Câncer da Boca* (Rapoport, A., ed.). Pancast, São Paulo, SP, Brazil, pp. 17-29.

Castaneda, F. and **Kinne, R.K.** (2001). Apoptosis induced in HepG2 cells by short exposure to millimolar concentrations of ethanol involves the Fas-receptor pathway. *J. Cancer Res. Clin. Oncol. 127*: 418-424.

Dave, B.J., Trivedi, A.H. and **Adhvaryu, S.G.** (1992). Role of areca nut consumption in the cause of oral cancers. A cytogenetic assessment. *Cancer 70*: 1017-1023.

Desai, S.S., Ghaisas, S.D., Jakhi, S.D. and **Bhide, S.V.** (1996). Cytogenetic damage in exfoliated oral mucosa cells and circulating lymphocytes of patients suffering from precancerous oral lesions. *Cancer Lett. 109*: 9-14.

Evans, H.J. (1997). Historical perspectives on the development of the *in vitro* micronucleus test: a personal view. *Mutat. Res. 392*: 5-10.

Ewing, J.A. (1984). Detecting alcoholism. The CAGE questionnaire. *J. Am. Med. Assoc. 252*: 1905-1907.

Franco, E.L., Kowalski, L.P., Oliveira, B.V., Curado, M.P., Pereira, R.N., Silva, M.E., Fava, A.S. and **Torloni, H.** (1989). Risk factors for oral cancer in Brazil: a case-control study. *Int. J. Cancer 43*: 992-1000.

Garewal, H.S., Ramsey, L., Kaugars, G. and **Boyle, J.** (1993). Clinical experience with the micronucleus assay. *J. Cell. Biochem. 17* (Suppl. F): 206-212.

Gattás, G.J.F. and **Saldanha, P.H.** (1993). Avaliação de alcoolismo *a posteriori*: validação de questionários padronizados. *37° Simpósio Internacional sobre a Prevenção e o Tratamento de Alcoolismo* (Abstract), São Paulo, SP, Brazil, Agosto, 2-6.

Gattás, G.J.F. and **Saldanha, P.H.** (1997). Chromosomal aberrations in peripheral lymphocytes of abstinent alcoholics. *Alcohol. Clin. Exp. Res. 21*: 238-243.

Gattás, G.J.F., Wajntal, A. and **Saldanha, P.H.** (1989). *In vitro* cytogenetic effects of cannabidiol on human lymphocyte cultures. *Rev. Bras. Genet. 12*: 613-623.

Ghose, U.R. and **Parida, B.B.** (1995). Cytological study of exfoliated buccal mucosa cells of tribes in Orissa State (India) with high risk for oral cancer. *Indian J. Cancer 32*: 95-99.

Hastak, K., Lubri, N., Jakhi, S.D., More, C., John, A., Ghaisas, S.D. and **Bhide, S.V.** (1997). Effect of turmeric oil and turmeric oleoresin on cytogenetic damage in patients suffering from oral submucous

fibrosis. *Cancer Lett. 116*: 265-269.
Heddle, J.A., Hite, M., Kirkhart, B., Mavournin, K., MacGregor, J.T., Newell, G.W. and **Salamone, M.F.** (1983). The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res. 123*: 61-118.
Kabat, G.C., Chang, C.J. and **Wynder, E.L.** (1994). The role of tobacco, alcohol use, and body mass index in oral and pharyngeal cancer. *Int. J. Epidemiol. 23*: 1137-1144.
Kayal, J.J., Trivedi, A.H., Dave, B.J., Nair, J., Nair, U.J., Bhide, S.V., Goswami, U.C. and **Adhvaryu, S.G.** (1993). Incidence of micronuclei in oral mucosa of users of tobacco products singly or in various combinations. *Mutagenesis 8*: 31-33.
Kumar, V., Rao, N.N. and **Nair, N.S.** (2000). Micronuclei in oral squamous cell carcinoma. A marker of genotoxic damage. *Indian J. Dent. Res. 11*: 101-106.
Li, N., Sun, Z., Han, C. and **Chen, J.** (1999). The chemopreventive effects of tea on human oral precancerous mucosa lesions. *Proc. Soc. Exp. Biol. Med. 220*: 218-224.
Lippman, S.M., Peters, E.J., Wargovic, M.J., Stadnyk, A.N., Dixon, D.O., Dekmezian, R.H., Loewy, J.W., Morice, R.C., Cunningham, J.E. and **Hong, W.K.** (1990). Bronchial micronuclei as a marker of an early stage of carcinogenesis in the human tracheobronchial epithelium. *Int. J. Cancer 45*: 811-815.
Llewellyn, C.D., Jonhson, N.W. and **Warnakulasuriya, K.A.** (2001). Risk factors for squamous cell carcinoma of the oral cavity in young people - a comprehensive literature review. *Oral Oncol. 37*: 401-418.
Lofti, C.F.P. and **Machado-Santelli, G.M.** (1996). Comparative analysis of colchicine induced micronuclei in different cell types *in vitro*. *Mut. Res. 349*: 77-83.
MacFarlane, G.J., Zheng, T., Marshall, J.R., Boffetta, P., Niu, S., Brasure, J., Merletti, F. and **Boyle, P.** (1995). Alcohol, tobacco, diet and the risk of oral cancer: a pooled analysis of three case-control studies. *Oral Oncol. Eur. J. Cancer 31B*: 181-187.
Maier, H., Dietz, A., Gewelke, U., Heller, W.D. and **Weidauer, H.** (1992). Tobacco and alcohol and the risk of head and neck cancer. *Clin. Invest. Med. 70*: 320-327.
Nakano, T. and **Oka, K.** (1991). Transition of Ki-67 index of uterine cervical tumors during radiation therapy. *Cancer 68*: 517-523.
Obe, G. and **Natarajan, A.T. (**1990). *Chromosomal Aberrations*. Springer-Verlag, Berlin, Germany.
Obe, G. and **Ristow, H.** (1979). Mutagenic, carcinogenic and teratogenic effects of alcohol. *Mutat. Res. 65*: 229-259.
Pindborg, J.J., Reibel, J., Roed-Petersen, B. and **Mehta, F.** (1980). Tobacco-induced changes in oral leukoplakic epithelium. *Cancer 45*: 2330-2336.
Prasad, M.P., Mukundan, M.A. and **Krishnaswamy, K.** (1995). Micronuclei and carcinogen DNA adducts as intermediate end points in nutrient intervention trial of precancerous lesions in the oral cavity. *Eur. J. Cancer Oral Oncol. 31B*: 155-159.
Ramirez, A. (2000). Analysis of metanucleated cells from alcoholics bearing oral carcinomas. *Genet. Mol. Biol. 23*: 504-505 (Thesis Abstracts).
Ramirez, A., Gattás, G.J.F., Carvalho, M.B., Rapopport, A. and **Saldanha, P.H. (**1999). Clinical implications of micronuclei frequency as a biomonitor for alcoholic patients with oral carcinomas. In: *Oral Oncology* (Varma, A.K., ed.). Vol VI. MacMillan, New York, NY, USA, pp. 199-204.
Roberts, D.M. (1997). Comparative cytology of the oral cavities of snuff users. *Acta Cytol. 41*: 1008-1014.
Sarto, F., Finotto, S., Giacomelli, L., Mazzotti, D., Tomanin, R. and **Levis, A.G.** (1987). The micronucleus assay in exfoliated cells of the human buccal mucosa. *Mutagenesis 2*: 11-17.
Seitz, H.K., Poschl, G. and **Simannowski, U.A.** (1998). Alcohol and cancer. *Recent Dev. Alcohol. 14*: 67-95.
Slomiany, A.L., Piotrowski, J., Piotrowski, E. and **Slomiany, A.** (1998). Induction of buccal mucosal apoptosis with chronic alcohol ingestion. *Biochem. Mol. Biol. Int. 44*: 381-389.
Stich, H.F. and **Rosin, M.P.** (1983). Quantitating the synergistic effects of smoking and alcohol consumption with the micronucleus test on human buccal mucosa cells. *Int. J. Cancer 31*: 305-308.
Stich, H.F. and **Rosin, M.P.** (1984). Micronuclei in exfoliated human cells as a tool for studies in cancer risk and cancer intervention. *Cancer Lett. 22*: 241-253.
Stich, H.F., Curtis, J.R. and **Parida, B.B.** (1982a). Application of the micronucleus test to exfoliated cells of high cancer risk groups: tobacco chewers. *Int. J. Cancer 30*: 553-559.
Stich, H.F., Stich, W. and **Parida, B.B.** (1982b). Elevated frequency of micronucleated cells in the buccal mucosa of individuals at high risk for oral cancer: betel quid chewers. *Cancer Lett. 17*: 125-134.
Stich, H.F., Rosin, M.P. and **Vallejera, M.O.** (1984). Reduction with vitamin A and beta-carotene administration of proportion of micronucleated buccal mucosal cells in Asian betel nut and tobacco chewers. *Lancet 2*: 1204-1206.
Stich, H.F., Rosin, M.P., Horney, A.P., Mathew, B., Sankaranarayanan, R. and **Nair, M.K.** (1988).

Remission of oral leukoplasias and micronuclei in tobacco-betel quid chewers treated with beta-carotene and with beta-carotene plus vitamin A. *Int. J. Cancer 42*: 195-199.

Tan, I.B., Roodenburg, J.L., Copper, M.P., Coebergh, J.W. and **van der Waal, I.** (2001). Early diagnosis and prevention of malignant tumors in the head and neck region. *Ned. Tijdschr. Geneeskd. 145*: 567-572.

Tolbert, P.E., Shy, C.M. and **Allen, J.W.** (1991). Micronuclei and other nuclear anomalies in buccal smears: a field-test in snuff users. *Am. J. Epidemiol. 134*: 840-850.

Tolbert, P.E., Shy, C.M. and **Allen, J.W.** (1992). Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutat. Res. 271*: 69-77.

Wu, D. and **Cederbaum, A.I.** (2001). Removal of glutathione produces apoptosis and necrosis in HepG2 cells overexpressing CYP2E1. *Alcohol. Clin. Exp. Res. 25*: 619-628.

Wyllie, A. (1981). Cell death: a new classification separating apoptosis from necrosis. In: *Cell Death in Biology and Pathology* (Bowen, I. and Lockshin R., eds.). Chapman and Hall, London, England, pp. 1-34.